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fraction was the dominant fraction in heart and kidney tissues whereas LD_5 was the preponderant fraction in skeletal muscle and liver. The serum of rhesus monkeys like man, showed the LD_2 fraction to be greater than the LD_1 fraction. All the other species tested showed no significant difference between LD_1 and LD_2 fractions. In man and in the rhesus monkey, but not the later species when $LD_1 > LD_2$ myocardial injury would be suggested. No CK-MB fraction was seen in the sera in any of the species tested, although many organs other than the heart contained high values of the CK-MB fraction. Isoenzyme electrophoresis may be a useful tool for evaluating single organ pathology in animals but diagnostic concepts in human diagnosis may not be applicable to laboratory animals.

Isoenzymes in Laboratory Animals

by

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In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," as promulgated by the Committee on the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council. The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

The views of the authors do not purport to reflect the positions of the Department of the Army or the Department of Defense.

ABSTRACT

Isoenzyme studies are useful in resolving the specific organ of origin when certain enzymes common to multiple organs are elevated in a disease process. In order to discriminate the specific organ effects of some toxins we determined the isoenzyme distribution patterns of lactate dehydrogenase (LD) and creatine kinase (CK) in monkeys, guinea pigs, rats, and mice. Serum and tissue extracts from normal animals were separated by electrophoresis. An immunochemical method of isoenzyme determination was used on mouse heart extracts. Five distinct bands of LD were demonstrated in all the species studied. As in man, LD_1 fraction was the dominant fraction in heart and kidney tissues whereas LD_5 was the preponderant fraction in skeletal muscle and liver. The serum of rhesus monkeys like man, showed the LD_2 fraction to be greater than the LD_1 fraction. All the other species tested showed no significant difference between LD_1 and LD_2 fractions. In man and in the rhesus monkey but not the later species when $LD_1 > LD_2$ myocardial injury would be suggested. No CK-MB fraction was seen in the sera in any of the species tested, although many organs other than the heart contained high values of the CK-MB fraction. Isoenzyme electrophoresis may be a useful tool for evaluating single organ pathology in animals but diagnostic concepts in human diagnosis may not be applicable to laboratory animals.

Circulating enzymes released from cells in the non-disease state maintain fairly constant activities in the serum at much lower concentrations than those which are found intracellularly[1]. Body organs and tissues have varying activities of these different enzymes, these being the basis for the so-called organ specific enzyme patterns. With tissue injury such as seen in infection, anoxia and infarction, the release of these intracellular enzymes is increased and an elevation of their serum activities will occur. In situations where only single organs have been injured, this will aid in the identification of the site of the disease process. However, two or more organs may contain similarly high amounts of the same enzymes, thus the ability for discriminating on the basis of enzymes alone may be lost. Isoenzymes will, however be helpful in differentiating organs whose isoenzyme percentage distribution patterns vary significantly.

Isoenzymes are distinct forms of the same enzymes. Although catalyzing the same reactions, they can be differentiated biochemically, physically, and immunochemically. In man, the isoenzyme patterns in different organs have been characterized. Physico-chemical methods such as heat treatment, or chemical inhibition have been used to differentiate the various fractions. The most widely used method for the separation of isoenzymes is electrophoresis, although column chromatography is becoming more popular. Many isoenzyme separation products are now commercially available for routine clinical use.

Isoenzyme studies are frequently utilized in human medicine and have become an important diagnostic modality[2,3]. Among the many known enzymes, the isoenzymes of lactate dehydrogenase (LD; EC1.1.1.27) and creatine phosphokinase (CK; EC2.7.3.2) have been the most utilized. They have daily applications in the diagnosis and evaluation of acute myocardial damage. Acute anoxic injury to the myocardium resulting in infarction is associated with

elevations of both enzymes, presence of the MB fraction of CK, and a characteristic LD fraction 1 greater than LD fraction 2, a reversal of the usual pattern[4]. The isoenzymes of alkaline phosphatase (ALP; EC 3.1.3.1) have also been utilized in the evaluation of liver, bone, and intestinal diseases[5,6]. Many other isoenzyme systems have not yet been fully utilized. The earlier expectation of using enzymes as specific organ markers has never been fully realized.

Textbooks of veterinary clinical pathology deal only briefly with the subject of isoenzymes. These tests have not been regularly utilized in veterinary medicine and or in basic research involving animals. We have determined the isoenzyme patterns for LD and CK in laboratory animals commonly used in our institution in an effort to establish reference values and determine whether isoenzymes can be used as markers of specific organ injury in laboratory animals

MATERIALS AND METHODS

Animals [(mouse,BALB/c), (rat, Fisher), (guinea pig, Hartley),] were sacrificed by cervical dislocation or CO_2 inhalation. Monkey (Cynomolgus and Rhesus) tissue was obtained from animals that died of natural causes. Serum from rabbits (NZW) was obtained by ear bleeding of normal animals. Various organs were immediately dissected and about one gram of tissue was homogenized in 2 ml of cold phosphate buffered saline 0.1 m pH 7.2 using a Polytron homogenizer[a]. The homogenates were spun at 10,000 x g for 10 minutes and the clarified supernatants were immediately used in the electrophoresis and immunochemical assays.

LD Electrophoresis

Total LD enzyme activity was determined in the supernatants using a COBAS analyzer[b]. Isoenzymes were electrophoretically separated using the Corning

Universal Agarose gel[c] with Corning Universal Barbital Buffer. Separation was carried out for 35 minutes in the Corning Electrophoresis Cell and Power Supply. The gels were overlaid with a colorimetric LD substrate, and incubated for 20 minutes at 37°C in a humid chamber. After a 20 minute wash in an agitated chamber with PBS 0.1 m pH 7.2, the gels were dried in a 70°C oven. The bands were scanned at 540 nm with a Beckman DU-8 spectrophotometer[d]. Areas of enzyme activity were calculated by a program contained in the instrument.

CK Electrophoresis

After a total CK activity was determined in a COBAS Analyzer, the isoenzymes were electrophoresed according to the Corning System as above using the Corning Special Purpose Electrophoresis Film agarose, 4-morpholinopropanesulfonic acid buffer (50 mmol/L pH 7.8, at 25°C), and Corning CK isoenzyme substrate. The separation was carried out for 20 minutes. The gels were immediately dried after incubation with substrate. Isoenzyme bands were scanned at 320 nm with the Beckman DU-8 Spectrophotometer.

Immunochemical Methods

The Isomer LD Kit[e] was used. Sample and reagent volumes were modified for economy, but the proportions were maintained. Enzyme activity was measured in the Cobas Analyzer. Briefly, a sample is reacted with goat anti-subunit M in antibody. Donkey anti-goat immunoglobulin attached to a polymer is added and centrifuged to precipitate all the M subunit containing isoenzymes.

Nomenclature

The numerical nomenclature was used for the LD isoenzymes as recommended by the IUPAC-IUB Commission on Biochemical Nomenclature (7). For CK isoenzymes, the more commonly used subunit designation was used.

Results

LD: The serum samples and tissue extracts from all the laboratory animals studied showed five distinct bands on LD electrophoresis. The isoenzyme fractions, although not aligning with human LD isoenzyme bands displayed similar anodic migration of all fractions except for the cathodic movement of LD_5 in the monkeys and rat (Figure 1). Only in the rhesus monkey was $LD_2 > LD_1$ and in all other species tested it was not. LD_5 fraction was the most dominant fraction in the mouse and rat in contrast to the relatively even distribution in the other species. Red blood cell hemolysates from rats and mice had LD_4 and LD_5 as the dominant fractions, and the cynomolgus and rhesus monkeys had LD_2 and LD_3 as the dominant fractions (Table 3). The LD_1 is the most dominant isoenzyme in heart and kidney tissues in all species, whereas skeletal muscle and liver tissues had LD_5 as the most abundant fraction and were low in LD_1.

CK: Electorphoresis patterns from the animals studied displayed 3 distinct isoenzyme bands. Minor, smaller bands with cathodic migration corresponding to the atypical CK and the mitochondrial CK^8 in man were seen in some tissues. The serum pattern in the non-human primates was identical to humans where only the MM band is seen. No MB band was seen in all the sera tested (Table 1) although non primates demonstrated a BB fraction in high concentrations. Among the tissues extracts, the MB fraction was seen in heart, skeletal muscle, and small intestines consistently. No CK activity was demonstrated in red blood cell hemolysates.

Immunochemical Method: The goat and M subunit antisera reacted with the M subunit of the mouse heart extract. All the M subunit containing isoenzymes (LD_2 to LD_4) were completely removed (Fig 2).

Discussion

The isoenzymes of lactate dehydrogenase and creatine phosphokinase among laboratory animals can be separated using presently available commercial reagents used for human specimens. Although there are differences between published electrophoretic data on humans and among the various species tested, some animal tissues displayed comparable isoenzyme distribution patterns. Variation among species, however, is expected and has been reported[9]. Reversals in the pattern of migration have even been documented[10].

Since no known control sera is available and no reference data on isoenzyme patterns in these animal species have been published, we tried to adhere to the numerical nomenclature as recommended by the IUPAC-IUB commission on Biochemical Nomenclature[7]. This also corresponds to the commonly used nomenclature in published literature. For CK we used the subunit designation instead of the numerical designation to facilitate comparison with human patterns.

As in man, heart and kidney tissues showed the highest activities of LD_1 and low activities of LD_5. In contrast most skeletal muscle and liver tissues contained LD_5 as the dominant fraction. It is then possible to discriminate skeletal muscle and liver tissues from heart and kidney. Hemolysis in specimens is a problem in humans since human erythrocytes are abundant in LD_1. Red cell hemolysates from animal species tested displayed different patterns. Rat and mouse red cell isoenzymes were abundant in the more cathodic fractions with $LD_4 > LD_5 > LD_3$. Monkeys displayed a pattern similar to man with more abundant anodic fractions with $LD_3 > LD_2 > LD_1$.

The LD_1 greater than LD_2 is specific for myocardial injury in man provided that neither renal infarction or hemolysis are present[4]. When seen in the presence of CK-MB band this pattern is virtually diagnostic of acute myocardial injury. LD_1 exceeded LD_2 in the serum of all species tested except the rhesus monkeys. The diagnostic usefulness of the reversed pattern (LD_1 greater than LD_2) as an indicator of myocardial injury is therefore invalid in these species.

The presence of the MB fraction in human serum is highly suggestive of myocardial injury in the proper clinical setting[4]. Although no CK-MB band was demonstrated in the animal sera tested, several animal tissues had high concentrations of this fraction. CK-MB fraction was consistently demonstrated in heart as well as skeletal muscle and small intestine. Other tissues also displayed the MB fraction, but were not consistent in all the species evaluated. More and more reports have been published of circulating MB fraction attributed to non-cardiac origin in neuromuscular disorders[10,11,12,13,14,15] and in healthy individuals after vigorous exercise[16,17]. In the event of multi-organ pathology such as in shock and trauma, these findings will make it difficult to interpret the significance of the presence of CK-MB fraction.

Two methods of isoenzyme determination were tried, the electrophoretic method which allowed demonstration of all the isoenzyme fractions, and the immunochemical method which gave isoenzyme fraction activity after unwanted fractions were removed. The electrophoretic method, although more tedious, was preferred and is recommended especially in initial studies with a particular animal species. There was early concern that the immunologic differences between species would prevent the human reagents from being useful for animal studies. Chilson has however concluded that M subunit from different species are quite similar[18]. Anti-M subunit antisera have been shown to cross-react

with M subunit from various species, but not with the H subunit[19] perhaps because the H subunit seems to be a poorer immunogen[20]. The LD reagents (Figure 1) used in mouse and rat heart tissue gave satisfactory results. The manufacturer's recommendations on diluting samples with very high enzyme activity must be adhered to so that all the precipitated fractions will be removed. The test relies on inhibition or precipitation of unwanted fractions and non-specific enzyme activity of the remaining reactions is measured. Since these fractions all catalyze the same reaction, the immunological method will not discriminate among the individual isoenzyme fractions, unlike the electrophoretic method where all the fractions are visually observed.

It is recommended that reference values and patterns be established for a particular animal used in the experiments. Variables in the methods used in different laboratories, animal to animal differences between suppliers, instrumental calibration and reagents may contribute to differences in patterns from laboratory to laboratory.

We have shown that various animal tissues can be differentiated by the use of isoenzymes. The presently available commercial products are adequate for this purpose. Concepts that are useful in humans such as the criteria for diagnosing myocardial infarction may not be applicable to other non-human species. In non-generalized or selective organ injuries, isoenzymes appear to be a useful tool for the evaluation of the pathologic process.

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Legend

Fig. 1 The agarose gel electrophoresis mobilities of LD isoenzymes in the human serum and other animal species.

Fig. 2 LD electrophoresis of mouse heart extract before (top) and after (bottom) treatment with goat antihuman M sub-unit antisera. Only the H homotetramer activity reamains.

Footnotes

[a] Brinkman Instruments, Westbury, NY

[b] Roche Analytical Instruments Inc., Nutley, NJ

[c] Corning, Palo Alto, CA

[d] Beckman Instruments Inc, Fullerton, CA

[e] Roche Diagnostics, Nutley, NJ

Table 1 LD isoenzymes in the serum of Laboratory animals (Percent Mean ± S.D.)

	LD_1	LD_2	LD_3	LD_4	LD_5
Cynomolgus Monkey	26 ± 3	25 ± 4	18 ± 3	15 ± 9	15± 2
Rhesus Monkey	23 ± 3	32 ± 3	23 ± 3	12 ± 2	10 ± 3
Rabbit (NZW)	13 ± 2	14 ± 1	26 ± 3	32± 3	15 ± 5
Guinea Pig (Hartley)	17	15	20	28	20
Rat (Fisher)	6 ± 4	3 ± 2	6 ± 4	16 ± 9	69 ± 15
Mouse (BALB/c)	3 ± 3	3 ± 3	7 ± 3	15 ± 5	70 ± 5

Table 2 CK isoenzymes in the serum of laboratory animals (Percent Mean ± S.D.)

	MM	MB	BB
Cynomolgus Monkey	100	0	0
Rhesus Monkey	100	0	0
Rabbit (NZW)	56 ± 4	0	44 ± 4
Guinea Pig (Hartley)	65	0	35
Rat (Fisher)	34 ± 3	0	66 ± 3
Mouse (BALB/c)	82 ± 5	0	18 ± 5

Table 3 LD isoenzymes in red blood cell hemolysates (Percent Mean ± S.D.)

	LD_1	LD_2	LD_3	LD_4	LD_5
Cynomolgus Monkey	16 ± 2	29 ± 1	48 ± 1	8 ± 3	0
Rhesus Monkey	12 ± 3	21 ± 1	53 ± 4	3 ± 1	6 ± 1
Rat (Fisher)	0	0	5 ± 2	63 ± 6	32 ± 4
Mouse (BALB/c)	0	0	7 ± 3	55 ± 8	38 ± 6

Table 4 LD isoenzymes in mouse tissues (Percent Mean ± S.D.)

	LD_1	LD_2	LD_3	LD_4	LD_5
Brain	33 ± 2	21 ± 3	20 ± 1	21 ± 4	5 ± 3
Heart	32 ± 3	25 ± 4	22 ± 3	14 ± 5	7 ± 3
Lung	7 ± 2	18 ± 1	25 ± 2	25 ± 6	23 ± 3
Liver	0	8 ± 8	10 ± 14	43 ± 15	40
Kidney	27 ± 3	19 ± 1	20 ± 4	19 ± 1	15 ± 4
Small Intestine	9 ± 3	14 ± 2	22 ± 4	16 ± 2	39 ± 3
Skeletal Muscle	15 ± 2	15 ± 3	20 ± 1	15 ± 4	35 ± 2

Table 5 CK isoenzymes in mouse tissues (Percent Mean ± S.D.)

	MM	MB	BB
Brain	53 ± 6	0	47 ± 6
Heart	71 ± 3	23 ± 3	6 ± 1
Lung	56 ± 6	5 ± 6	39 ± 2
Liver	63 ± 3	0	37 ± 3
Kidney	67 ± 10	0	33 ± 9
Small Intestine	44 ± 6	26 ± 6	30 ± 7
Skeletal Muscle	86 ± 8	4 ± 6	10 ± 3
Red Blood Cell	0	0	0

Table 6 Distribution of LD isoenzymes in rat tissues (Percent Mean ± S.D.)

	LD_1	LD_2	LD_3	LD_4	LD_5
Brain	33 ± 2	22 ± 3	25 ± 1	20 ± 4	0
Heart	28 ± 8	25 ± 4	22 ± 4	19 ± 1	7 ± 6
Lung	15 ± 1	20 ± 4	27 ± 4	24 ± 2	16 ± 4
Liver	0	2 ±	10 ±2	22 ± 11	66 ± 14
Kidney	24 ± 6	17 ± 4	23 ± 1	17 ± 7	10 ± 6
Spleen	3 ± 1	20 ± 6	33 ± 11	31 ± 8	15 ± 9
Small Intestine	0	13 ± 4	19 ± 2	23 ± 3	46 ± 3
Skeletal Muscle	8 ± 4	14 ±	15 ± 5	34 ± 11	30

Table 7 Distribution of CK isoenzymes in rat tissues
(Percent Mean ± S.D.)

	MM	MB	BB
Brain	66 ± 15	0	34 ± 15
Heart	55 ± 1	29 ± 2	16 ± 2
Lung	42 ± 1	18 ± 6	40 ± 8
Liver	54 ± 20	0	46 ± 20
Kidney	47 ± 5	0	54 ± 4
Spleen	10 ± 2	0	90 ± 5
Small Intestine	42 ± 3	24 ± 9	34 ± 4
Skeletal Muscle	68 ± 6	23 ± 3	9 ± 3

Table 8 LD isoenzymes in guinea pig tissues (Mean ± S.D.)

	LD_1	LD_2	LD_3	LD_4	LD_5
Brain	30 ± 4	27 ± 2	25 ± 1	15 ± 3	3 ± 2
Heart	41 ± 5	25 ± 3	19 ± 4	12 ± 2	3 ± 2
Lung	22 ± 6	25 ± 3	25 ± 2	18 ± 1	10 ± 4
Liver	6 ± 2	11 ±	28 ± 6	34 ± 5	21 ± 9
Kidney	38 ± 4	23 ± 3	19 ±	14 ± 3	6 ± 2
Spleen	15 ± 2	19 ± 1	30 ± 5	24 ± 6	12 ± 2
Small Intestine	16 ± 3	29 ± 4	20 ± 2	20 ± 3	15 ± ?
Skeletal Muscle	8 ± 1	5 ± 2	11 ± 2	22 ± 6	54 ± 5

Table 9 CK isoenzymes in guinea pig tissues (Mean ± S.D.)

	MM	MB	BB
Brain	54 ± 2	0	46 ± 7
Heart	66 ± 3	24 ± 3	10 ± 2
Lung	47 ± 3	21 ± 6	32 ± 3
Liver	39 ± 4	25 ± 8	36 ± 3
Kidney	49 ± 6	23 ± 9	28 ± 3
Spleen	31 ± 3	22 ± 7	47 ± 2
Small Intestine	48 ± 4	26 ± 3	26 ± 5
Skeletal Muscle	79 ± 5	7 ± 5	12 ± 2

Table 10 LD isoenzymes in monkey tissues (Percent Mean ± S.D.)

	LD_5	LD_4	LD_3	LD_2	LD_1
Spleen	7 ± 1	20 ± 4	48 ± 8	20 ± 4	6
Kidney	1 ± 1	13 ± 2	27 ± 2	20 ± 4	40 ± 10
Brain	0	9 ± 1	33 ± 2	30 ± 3	23 ± 6
Liver	33 ± 1	39 ± 1	23 ± 3	5 ± 4	1 ± 2
Skeletal Muscle	46 ± 2	28 ± 1	13	8 ± 2	6
Lung	7 ± 1	18 ± 1	41 ± 4	28 ± 4	8
Heart	4 ± 2	13	26	18 ± 3	40 ±
Small Intestine	11 ± 1	22 ± 2	32 ± 1	21 ± 1	15 ±

Table 11 CK isoenzymes in monkey tissues (Percent Mean ± S.D.)

	MM	MB	BB
Brain	53	0	47
Small Intestine	48	26	26
Kidney	58	7	35
Liver	60	0	40
Lung	61	0	39
Heart	60	30	10
Spleen	40	0	60
Skeletal Muscle	85	10	5